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GlaxoSmithKline Biologicals s.a.
Rue de l'Institut 89, B-1330 Rixensart, , Belgium

Patents ADP number *(if you know it)* 81012710 01

If the applicant is a corporate body, give the country/state of its incorporation

Belgium
see continuation sheet for further applicant(s)

4. Title of the invention — Vaccine Composition

5. Name of your agent *(if you have one)*

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M J Lubienski

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VACCINE COMPOSITION

FIELD OF THE INVENTION

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are less phase variable in terms of their LOS immunotype, and from which novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines can be derived.

BACKGROUND OF THE INVENTION

Neisseria meningitidis (meningococcus) is a Gram negative bacterium frequently isolated from the human upper respiratory tract. It is a cause of serious invasive bacterial diseases such as bacteremia and meningitis. The incidence of meningococcal disease shows geographical, seasonal and annual differences (Schwartz, B., Moore, P.S., Broome, C.V.; Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). The bacterium is commonly classified according to the serogroup if its capsular polysaccharide.

Most disease in temperate countries is due to strains of serogroup B and varies in incidence from 1-10/100,000/year total population - sometimes reaching higher values (Kaczmarski, E.B. (1997), Commun. Dis. Rep. Rev. 7: R55-9, 1995; Scholten, R.J.P.M., Bijlmer, H.A., Poolman, J.T. et al. Clin. Infect. Dis. 16: 237-246, 1993; Cruz, C., Pavez, G., Aguilar, E., et al. Epidemiol. Infect. 105: 119-126, 1990).

Epidemics dominated by serogroup A meningococci, mostly in central Africa, sometimes reach incidence levels of up to 1000/100,000/year (Schwartz, B., Moore, P.S., Broome, C.V. Clin. Microbiol. Rev. 2 (Supplement), S18-S24, 1989). Nearly all cases as a whole of meningococcal disease are caused by serogroup A, B, C, W-135 and Y meningococci, and a tetravalent A, C, W-135, Y capsular polysaccharide vaccine is available (Armand, J., Arminjon, F., Mynard, M.C., Lafaix, C., J. Biol. Stand. 10: 335-339, 1982).

The frequency of *Neisseria meningitidis* infections has risen in the past few decades in many European countries. This has been attributed to increased transmission due to an increase in social activities (for instance swimming pools, theatres, etc.). It is no longer uncommon to isolate *Neisseria meningitidis* strains that are less sensitive or resistant to some of the standard antibiotics. This phenomenon

has created an unmet medical need and demand for new anti-microbial agents, vaccines, drug screening methods, and diagnostic tests for this organism.

The available polysaccharide vaccines are currently being improved by way of chemically conjugating them to carrier proteins (Lieberman, J.M., Chiu, S.S., Wong, V.K., et al. JAMA 275 : 1499-1503, 1996).

A serogroup B vaccine, however, is not available. The serogroup B capsular polysaccharide has been found to be nonimmunogenic - most likely because it shares structural similarity with host components (Wyle, F.A., Artenstein, M.S., Brandt, M.L. et al. J. Infect. Dis. 126: 514-522, 1972; Finne, J.M., Leinonen, M., Mäkelä, P.M. Lancet ii.: 355-357, 1983). Effort has therefore been focused in trying to develop serogroup B vaccines from outer membrane vesicles (or blebs) or purified protein components therefrom.

Alternative meningococcal antigens for vaccine development are meningococcal lipooligosaccharides (LOS). These are outer membrane bound glycolipids which differ from the lipopolysaccharides (LPS) of the Enterobacteriaceae by lacking the O side chains, and thus resemble the rough form of LPS (Griffiss et al. Rev Infect Dis 1988; 10: S287-295). Heterogeneity within the oligosaccharide moiety of the LOS generates structural and antigenic diversity among different meningococcal strains (Griffiss et al. Inf. Immun. 1987; 55: 1792-1800). This has been used to subdivide the strains into 12 immunotypes. Immunotypes L3, L7, L9 have an identical carbohydrate structure and have therefore been designated L3,7,9. Meningococcal LOS L3,7,9, L2 and L5 can be modified by sialylation, or by the addition of cytidine 5'-monophosphate-N-acetylneuraminic acid. Antibodies to LOS have been shown to protect in experimental rats against infection and to contribute to the bactericidal activity in children infected with *N. meningitidis* (Griffiss et al J Infect Dis 1984; 150: 71-79).

A problem associated with the use of LOS in a meningococcal vaccine, however, is its toxicity (due to its Lipid A moiety).

LOS is also present on the surface of meningococcal blebs. For many years efforts have been focused on developing meningococcal outer membrane vesicle (or bleb) based vaccines (de Moraes, J.C., Perkins, B., Camargo, M.C. et al. Lancet 340: 1074-1078, 1992; Bjune, G., Hoiby, E.A. Gronnesby, J.K. et al. 338: 1093-1096, 1991). Such vaccines have the advantage of including several integral outer-membrane proteins in a properly folded conformation which can elicit a protective

immunological response when administered to a host. In addition, Neisserial strains (including *N. meningitidis* serogroup B - menB) excrete outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. More often, however, blebs are prepared by methods comprising a detergent (e.g. deoxycholate) extraction of the bacterial cells (e.g. EP 11243), which has the effect of removing a lot of the LOS from the vaccine. This is desired due to the toxicity of LOS (also called endotoxin) as described above.

A further problem with using LOS as a vaccine antigen is that 12 LPS immunotypes exist with a diverse range of carbohydrate-structures (M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021). Antibodies raised against one immunotype fail to recognise a different immunotype. Although effort has been focused on producing a generic "core" region of the oligosaccharide portions of the LOS immunotypes (e.g. WO 94/08021), the bactericidal activity of antibodies generated against the modified LOS is lost. Thus a vaccine may need to have many LOS components of different immunotype to be effective.

Even if a few immunotypes could be selected, a final problem exists. To make LOS (or blebs containing LOS) of a certain immunotype a meningococcal strain needs to be cultured. A feature of meningococcal LOS is the reversible, high frequency switching of expression (phase variation) of terminal LPS structures (M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021). The phase variation exhibited by the LOS is an obstacle to the development of a cross-protective OMV or subunit vaccine based on the use of LOS as a protective antigen. For MenB strain H44/76, for example, the rate of switching from L3 to L2 immunotype is estimated at 1 in 1000 to 5000. Antibodies raised against the L3 structure failed to recognize the L2 immunotype and *vice versa*. Therefore it is extremely hard to maintain a LOS or bleb production strain with a constant, homogenous LOS immunotype.

The present invention presents processes for ameliorating one or more of the above problems, and presents methods for making novel vaccines based on meningococcal LOS as a protective antigen.

SUMMARY OF THE INVENTION

The present invention relates to processes of making vaccine compositions for the effective prevention or treatment of neisserial, preferably meningococcal, disease. The processes of the invention involve making a genetically engineered meningococcal strain which has a fixed or locked LOS immunotype. In particular, methods are disclosed which allow L2 and L3 LOS immunotypes to be fixed. A process for making LOS or blebs from such engineered strains is further covered, as is a method of making an immunogenic composition comprising the steps of making the above LOS or blebs and mixing with a pharmaceutically acceptable excipient.

DESCRIPTION OF THE INVENTION

The subject matter of and information disclosed within the publications and patents or patent applications mentioned in this specification are incorporated by reference herein.

Reference to "lipooligosaccharide" (or "LOS") may also be referred to as "lipopolysaccharide" or "LPS".

A locus containing various *lgt* genes is required for the biosynthesis of the terminal LOS structure (the sequences of which are known in the art - see M. P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021 and references cited therein). Meningococci can change the immunotype of the expressed LOS via a mechanism of phase variable expression of some of these genes. The phase variable expression of LOS in L3 type *menB* strains (e.g. MC58, H44/76) operates via high frequency mutations in a homopolymeric G tract region of *lgtA*. The major difference between L2 and L3 immunotypes in those strains is the presence or absence of a glucose residue on the second heptose (fig. 1). The addition of this residue is catalyzed by the *lgtG* gene product, which also exhibits phase variable expression. Other strains (e.g. 126E) can switch its LPS saccharidic structure from an L3 to an L1 immunotype through the expression of a third phase variable *lgtC* gene that catalyzes the extension of an additional galactose (fig.2) (M.P. Jennings *et al*, Microbiology 1999, **145**, 3013-3021).

The present inventors have overcome this problem by developing methods of producing meningococcal vaccine production strains which are fixed (i.e. not phase variable) in their LOS immunotype. Thus in a first aspect the present invention

provides a process of making a genetically engineered meningococcal strain comprising the step of genetically engineering a meningococcal strain with phase-variable LOS expression, to render LOS expression non-phase variable (or fixed).

In a specific embodiment, the process results in non-phase variable LOS having exclusively an L2 immunotype (preferably constitutively expressed).

Preferably such a process has a genetic engineering step comprising the elements of fixing the expression of both the lgtA and lgtG gene products (i.e. such that expression of full-length, functional gene product may not be switched off by phase variation – the genes are constitutively expressed).

Clearly if either of the lgtA or lgtG genes is naturally fixed in a meningococcal strain to be used, only the gene that is still phase variable need be engineered.

Although fixing could take place by inserting extra copies either or both of the constitutively genes into the organism (whilst preferably inactivating the wild-type copy), this method is more convoluted than simply engineering the wild-type copy of the gene(s).

Preferably, the expression of either or both of lgtA and lgtG gene products is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the respective gene whilst maintaining the open-reading frame of the gene in frame.

For the homopolymeric G tract in the lgtA open-reading frame it is preferred that the tract is reduced to 8, more preferably 5, or most preferably 2 consecutive G nucleotides.

For the homopolymeric C tract in the lgtG open-reading frame it is preferred that the tract is reduced to 8, 5 or, most preferably, 2 consecutive C nucleotides.

Such tract reductions can be simply performed in general using homologous recombination (see WO 01/09350) between a plasmid construct containing the reduced tract and the genomic DNA of the strain to be changed after transformation of the strain with the plasmid.

Alternatively, the expression of lgtA gene product can be fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine (GGA, GGC or GGT), or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine (the final G being part

of the tract) is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame. For instance, a 5G homopolymeric tract can advantageously have one GGG Glycine codon be mutated to a nucleotide sequence GGG(A/C/T)G.

Furthermore, the expression of lgtG gene product can be alternatively fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: one or more CCC codons encoding Proline is changed to any other codon encoding Proline (CCA, CCG or CCT), or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine (the final CC pair being part of the tract) is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

It is preferred that in the above scenarios codons are replaced with codons encoding the same amino acids, however where conservative mutations are used it is preferred that: 1) codons are selected containing 2 or (preferably) fewer nucleotides of the type making up the tract, & 2) the new encoded amino acid is a conservative mutation. Conservation mutations are understood by skilled persons in this field. However preferred substitutions are detailed in the table below.

Original residue	Exemplary substitutions	Preferred substitution
A	V, L, I	V
G	P, A	A
P	A, G	A
S	T, A	T

In such a scenario it is preferred that 2, 3 or, more preferably, 4 codons in the homopolymeric tract are changed, most preferably to encode the identical amino acid.

A combination of the above methods of the invention (reducing the tract length and altering the tract's codon usage) could be used to fix the lgtA and/or lgtG genes. For instance by both reducing the lgtA tract to 5 G residues, and replacing one of the GGG codons encoding Glycine to one of the other 3 codons encoding Glycine [yielding a final tract nucleotide sequence of GGG(A/C/T)G].

In an advantageous embodiment the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open

reading frame of the respective gene to 2 or 5 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of lgtG gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: 1, 2 or preferably 3 CCC codons encoding Proline is changed to any other codon encoding Proline (CCA, CCG, or CCT), or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

In a further specific embodiment, the process of the invention results in non-phase variable LOS having exclusively an L3 immunotype (preferably constitutively expressed).

In this specification, all reference to "L3" immunotype will be a reference to "L3,7,9", "L3", "L7", and "L9" immunotypes which have identical carbohydrate structures.

In this process the genetic engineering step preferably comprises the elements of fixing the expression of the lgtA gene product such that expression of full-length, functional product may not be switched off by phase variation (i.e. is constitutively expressed as described above), and permanently downregulating the expression of functional gene product from the lgtG gene.

By "downregulating the expression of functional gene product" it is meant that additions, deletions or additions are made to the promoter or open reading frame of the gene such that the biosynthetic activity of the total gene product reduces (by 60, 70, 80, 90, 95 or most preferably 100%). Clearly frameshift mutations may be introduced, or weaker promoters substituted , however most preferably most or all of the open reading frame and/or promoter is deleted to ensure a permanent downregulation of the gene product. See WO 01/09350 for further methods of gene downregulation.

Clearly if lgtA expression is naturally fixed or lgtG expression is naturally down-regulated in a wild-type meningococcal strain to be altered, only the gene that is in need of change to fix the immunotype should be engineered.

Although fixing could take place by inserting extra copies of the constitutively expressed lgtA genes into the organism (whilst preferably inactivating the wild-type copy), this method is more convoluted than simply engineering the wild-type copy of the gene(s).

The expression of lgtA gene product can be fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene whilst maintaining the open-reading frame of the gene in frame (preferably the homopolymeric G tract in the lgtA open-reading frame is reduced to 8, more preferably 5 or, most preferably, 2 consecutive G nucleotides) and/or by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame (as described above). For instance, a 5G homopolymeric tract can advantageously have one GGG Glycine codon be mutated to a nucleotide sequence GGG(A/C/T)G.

A combination of the above methods of the invention (reducing the tract length and altering the tract's codon usage) could be used to fix the lgtA gene. For instance by both reducing the lgtA tract to 5 G residues, and replacing one of the GGG codons encoding Glycine to one of the other 3 codons encoding Glycine [yielding a final tract nucleotide sequence of GGG(A/C/T)G].

Preferably the expression of functional gene product from the lgtG gene is switched off (i.e. there is no or negligible lgtG gene product biosynthetic activity post mutation).

In an advantageous embodiment the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the respective gene to 5 or 2 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of functional gene product from the lgtG gene is switched off by deleting all or part of the promoter and/or open-reading frame of the gene.

Where the meningococcal strain to be altered has an lgtC gene (e.g. strain 126E), it is preferred that the processes of the invention have a genetic engineering step which comprises an element of permanently downregulating the expression of functional gene product from the lgtC gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

For potential safety reasons, the above processes can be extended. The safety of antibodies raised to L3 or L2 LPS has been questioned, due to the presence of a structure similar to the lacto-N-neotetraose oligosaccharide group present in human glycosphingolipids. Even if a large number of people has been safely vaccinated with deoxycholate extracted vesicle vaccines containing residual amount of L3 LPS (G. Bjune *et al*, Lancet (1991), **338**, 1093-1096; GVG. Sierra *et al*, NIPH ann (1991), **14**, 195-210), the deletion of the terminal part of the LOS saccharidic is advantageous in preventing any cross-reaction with structures present at the surface of human tissues. Inactivation of the *lgtB* gene results in an intermediate LPS structure in which the terminal galactose residue and the sialic acid are absent. Such intermediates could be obtained in an L3 (*lgtA* fixed on and *lgtG* fixed off) and an L2 (*lgtA* and *lgtG* fixed on) LPS strain. An alternative and less preferred (short) version of the LPS can be obtained by turning off the *lgtE* gene.

Therefore, the above processes may also have a genetic engineering step comprising the element of permanently downregulating the expression of functional gene product from the lgtB or lgtE gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene.

Where the process involves a wild-type meningococcus B strain, it is preferred that the genetic engineering step of the process comprises the element of permanently downregulating the expression of functional gene product from the siaD gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Such an inactivation is also described in WO 01/09350. The mutation is the most advantageous of many mutations that can result in removing the human-similar epitope from the capsular polysaccharide. This is because it is the only mutation that has no effect on the biosynthesis of LOS, and thus it is advantageous in a process which aims at ultimately

using LOS as a protective antigen. Most preferably the processes of the invention utilise a lgtB$^-$ siaD$^-$ meningococcus B mutant strain.

The processes of the invention may also include steps which render the LOS less toxic. Although this is not necessary for intranasal immunization with native OMV (J.J. Drabick *et al*, Vaccine (2000), **18**, 160-172), for parenteral vaccination detoxification would present an advantage. LOS can be detoxified genetically by mutation/modification/inactivation of genes involved in LipidA biosynthesis for example by downregulating the expression of functional gene product from the msbB and/or htrB genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter and/or open-reading frame of the gene. Alternatively (or in addition) one or more of the following genes may be upregulated (by introducing a stronger promoter or integrating an extra copy of the gene): pmrA, pmrB, pmrE and pmrF. See WO 01/09350 for more detail on the above detoxification methods, and for relevant promoter / gene sequences.

A further aspect of the invention is a process of isolating L2 LOS comprising the steps of producing a genetically engineered meningococcal strain with a fixed L2 immunotype by the process of the invention as described above, and isolating L2 LOS from the resulting strain. An additional advantageous step may be added to this process, namely conjugating the L2 LOS to a carrier comprising a source of T-cell epitopes (rendering the LOS an even better immunogen) and/or the step of presenting the L2 LOS in liposome formulations known in the art.

The process of isolation of LOS from bacteria is well known in the art (see for instance the hot water-phenol procedure of Wesphal & Jann [Meth. Carbo. Chem. 1965, 5:83-91]).

For the purposes of this invention "a carrier comprising a source of T-cell epitopes" is usually a peptide or, preferably, a polypeptide or protein. Conjugation techniques are well known in the art. Typical carriers include protein D from non typeable *H. influenzae*, tetanus toxoid, diphtheria toxoid, and CRM197.

Similarly a still further aspect of the invention is a process of isolating L3 LOS comprising the steps of producing a genetically engineered meningococcal strain with a fixed L3 immunotype by the process of the invention as described above, and isolating L3 LOS from the resulting strain. An additional advantageous step may be

added to this process, namely conjugating the L3 LOS to a carrier comprising a source of T-cell epitopes and/or the step of presenting the L3 LOS in a liposome formulation.

A further aspect of the invention is a process of isolating meningococcal blebs having an L2 or L3 LOS immunotype, comprising the steps of producing a genetically engineered meningococcal strain with a fixed L2 or L3 immunotype, respectively, by the processes of the invention as described above; and isolating blebs from the resulting strain.

Outer Membrane Vesicles (OMVs or blebs) can be isolated by many known techniques (Fredriksen *et al*, NIPH Annals (1991), **14**, 67-79; Zollinger *et al*, J. Clin Invest (1979), **63**, 836-848; Saunders *et al*, Infect Immun (1999), **67**, 113-119; J.J. Drabick *et al*, Vaccine (1999), **18**, 160-172). These divide into 2 main groups – techniques which use deoxycholate (about 0.5%) to extract blebs from meningococcus, and techniques that use low levels of deoxycholate (DOC) or no deoxycholate at all. DOC free process blebs have the interesting feature of maintaining high level of LOS in the OMV – which is advantageous in a vaccine where LOS is a protective antigen. Compared to DOC extracted blebs, the concentration of L3 Ags in OMV obtained by a DOC free process is approximately ten times higher, also taking into account the fixing of *lgtA*. A detergent-free (preferably DOC-free) process of preparing blebs is preferred for the purposes of the processes of this invention for this reason, although extraction with a buffer containing low levels of detergent (preferably DOC) may also be advantageous in that the step would leave most of the tightly interacting LOS in the bleb whilst removing any more toxic loosely retained LOS. Preferably 0-0.3% detergent (preferably DOC) is used for bleb extraction, more preferably 0.05-0.2%, and most preferably around 0.1%. DOC free (or low DOC) extraction processes are particularly preferred where the LOS has been detoxified by one of the methods detailed above.

The above processes of the invention may comprise an additional advantageous step of conjugating the L2 or L3 LOS to an outer membrane protein (e.g. PorA or PorB) also present in the blebs (e. g. by the conjugation chemistry described in Devi *et al*, Infect Immun (1997), **65**, 1045-1052, or other treatments capable to create covalent links between molecules). Again this process advantageously enhances the stability and/or immunogenicity (providing T-cell help)

and/or antigenicity of the LOS antigen within the bleb formulation. One or more of Men A, C, Y or W capsular polysaccharides (preferably at least MenC) may also be conjugated onto an outermembrane protein of the bleb as well.

A process for making immunogenic compositions or vaccines are also provided comprising the steps of producing isolated L2 LOS by the process of the invention as described above and/or producing isolated meningococcal blebs having an L2 LOS immunotype by the processes of the invention as described above, and formulating the L2 LOS and/or blebs with a pharmaceutically acceptable excipient.

Likewise a process for making immunogenic compositions or vaccines are also provided comprising the steps of producing isolated L3 LOS by the process of the invention as described above and/or producing isolated meningococcal blebs having an L3 LOS immunotype by the processes of the invention as described above, and formulating the L3 LOS and/or blebs with a pharmaceutically acceptable excipient.

An advantageous process of the invention is a process of making a multivalent immunogenic composition or vaccine comprising the steps of producing one or both of isolated L2 LOS or isolated meningococcal blebs having an L2 LOS immunotype by the processes of the invention as described above, and producing one or both of isolated L3 LOS or isolated meningococcal blebs having an L3 LOS immunotype by the processes of the invention as described above, and mixing said L2 and L3 vaccine components together along with a pharmaceutically acceptable excipient. Preferably the process mixes isolated L2 and L3 LOS together which are made as described above (most preferably conjugated and in a liposome formulation). More preferably the process mixes L2 and L3 blebs together which are made as described above. Such compositions are advantageous as approximately 70% of meningococcus B immunotypes observed in disease isolates have an L3 structure, and 30% are L2. The invention therefore describes a process which can yield a universal meningococcus B vaccine.

The process of making immunogenic compositions or vaccines as described above may have an additional step of adding one or more (2, 3 or 4) meningococcal polysaccharides (either plain or conjugated to a carrier comprising T-cell epitopes) from serogroups A, C, Y or W to the composition. Preferably at least C is added (most preferably conjugated), and more preferably A and C or Y and C (preferably all

conjugated) and most preferably A, C, Y and W (preferably all conjugated). The term "polysaccharide" is intended to cover unsized or sized (reduced in size) polysaccharides, or sized oligosaccharides.

A further step that may be added to the above processes for making immunogenic compositions or vaccines as described above is the addition of a suitable adjuvant. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate (preferably aluminium hydroxide), but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be added include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A (or other non-toxic derivatives of LPS), and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) [or non toxic LPS derivatives] together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 [or other saponin] and 3D-MPL [or non toxic LPS derivative] as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 [or saponin] is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation that may be added. Other adjuvants that may be added comprise a saponin, more preferably QS21 and/or an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) may also be added

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

An immunoprotective dose of vaccines can be administered via the systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Typically bleb quantity in each vaccine dose is selected as an amount which induces an immunoprotective response

without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of each bleb, preferably 5-50μg, and most typically in the range 5 - 25μg.

Ghost or Killed Whole cell vaccines

The inventors envisage that the above processes concerning blebs can be easily extended to processes concerning ghost or killed whole cell preparations and vaccines (with identical advantages). Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. The processes concerning blebs described throughout this document are therefore applicable to the processes concening ghosts and killed whole cells for the purposes of this invention.

EXAMPLES

The examples below are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1: Making a fixed L3 strain (fixed lgtA)

Genes encoding glycosyltransferases in *Neisseria meningitidis* often contain simple tandem repeats (for example, homopolymeric tracts) which mediate phase variation (high frequency reversible on/off switching of gene expression (Jennings *et al* 1995, Mol Micro **18** 724; Jennings *et al* 1999, Microbiology **145** 3013). The repeated sequences in these genes are present in the open reading frame and are transcribed and translated into protein. Phase variation may be eliminated by reducing (in frame) the homopolymeric tract. An alternative approach to deletion of the repeat sequences is to alter the nucleotide sequence in the repeat region so that it encodes the same amino acid sequence but does not constitute a repeat (see *lgtG "fixed"* mutant, Example 3). In this work we sought to "fix" the expression of certain glycosyltransferase genes in *Neisseria meningitidis* so that their expression was constituitively "on" or "off". In this way the LPS antigen expressed could be fixed to a defined structure, no longer subject to phase variation.

Mutation of the *lgtA* gene of give constitutive expression of LgtA – the *lgtA2G* mutant

In order to fix the expression of the *lgtA* gene so that it was fixed "on" we altered the homopolymeric tract of the *lgtA* gene so that only 2 G residues remained in the homopolymeric tract region (the wild type strains has 14 G; Jennings *et al* 1995, *supra*). Using primers Lic31ext: 5'- CCT TTA GTC AGC GTA TTG ATT TGC G – 3' and lgtAG2 5'-ATC GGT GCG CGC AAT ATA TTC CGA CTT TGC CAA TTC ATC – 3' in PCR with *Neisseria meningitidis* strain MC58 chromosomal DNA as template we amplified the region to be altered. The latter primer incorporated the change in the *lgtA* sequence from 14G to 2G. The resulting PCR product was cloned

into pT7Blue (Novogen), to create plasmid pT7lgtAG2. To reconstitute to complete *lgtA* gene so that the plasmid could be used to transform the new allele into *Neisseria meningitidis*, a *Bss*HII fragment from plasmid p1B11 (Jennings *et al* 1995, *supra*) was cloned into the *Bss*HII site of pT7lgtAG2 in the correct orientation. Nucleotide sequence analysis confirmed the correct orientation of the gene and that the sequence segment was identical to the corresponding section of the wild-type *lgtA* gene (Genbank accession NMU25839) apart from the alteration of the homopolymeric tract from 14 to 2 G residues. Using a similar process, variants of the lgtAG2 primer mutations were made so that a series of similar plasmids were created that contained *lgtA* alleles with 3, 4, 5, 7 and 10 G residues in the homopolymeric tract region.

Using a similar process the phase variation of the homopolymeric tract can also be fixed by altering the poly G regions so that the GGG codons are replaced with alternative glycine codons arranged so that the same amino acid sequence is encoded, but the nucleotide sequence does not have a repetitive nature and is unlikely to phase vary (see lgtG example below). In addition, a combination of the 2 methods could also be used – for instance the homopolymeric tract could be cut to 5 G residues & a GGG codon replaced with an alternative glycine codon.

Transformation of strain MC58¢3 with pT7lgtAG2 to transfer the *lgtAG2* to the chromosome of *Neisseria meningitidis* strain MC58¢3

In order to transfer the *lgtAG2* mutation to the chromosome of *Neisseria meningitidis* to make a mutant strain, the plasmid pT7lgtAG2 was linearized and used to transform *Neisseria meningitidis* strain MC58¢3 containing an *lgtA::kan* mutation (Jennings *et al* 1995, *supra*). Positive colonies were detected by mAb 4A8B2 in colony-immunoblot (Jennings *et al* 1999, *supra*). Confirmation that the LgtA positive phenotype (L3 immunotype structure) of the transformants was the result of the transfer of the *lgtAG2* allele to the chromosome was confirmed by PCR of the relevant section of the *lgtA* gene using primers Lic31 ext and Lic16ext: 5'- CGA TGA TGC TGC GGT CTT TTT CCA T -3', followed by nucleotide sequencing with the same set of primers. The resulting strain 2G2 had the genotype: MC58 parent strain; *siaD::ery lgtAG2*). Strain 2G2 was subsequently transformed with the a plasmid containing an *lgtB::kan* mutation (Jennings *et al* 1995, *supra*) to create strain

2G2ecoNI, this strain had the genotype: MC58 parent strain; *siaD::ery lgtAG2 lgtB::kan*

Example 2: Experiments with fixed L3 and intermediate (lgtB⁻) DOC free blebs (non-detoxified LOS) induced cross-bactericidal antibodies

The MC58 derivative strain used is B:P1.7.16, *opc-*, *siaD-*. This strain was genetically modified to express either L3 (strain 2G2 [modified to reduce the homopolymeric tract to only 2 G nucleotides], *lgtA* fixed on) or an intermediate epitope (strain 2G EcoN1b-1, *lgtA* fixed on as with 2G2 but *lgtB* additionally turned off) or an LPS in short version (strain C6, *lgtE* off). OMV were produced according either a DOC process or DOC free process.

Mice (10 per group) were immunized three times by the intra-muscular route on Day 0, 20 and 28. They received 1 or 10 μg (protein content) of blebs formulated on Al(OH)3. Blood samples were taken on day 28 (post II) and day 42 (post III).

Bactericidal assays were done on pooled sera and using homologous strains (MC58 and H44/76) and two heterologous strains (M97250687 and M9725078) with baby rabbit serum as source of exogenous complement.

The following table summarizes the results (bactericidal titers for 50% killing):

Antigen	Blood samples	Strain and serotype: MC58 P1.7.16	H44/76TT P1.7.16	M97250687 P1.19.15	M97252078 P1.4
c6 no doc 10ug IM	Post II	>2560	>2560	>2560	98
c6 no doc 10ug IM	Post III	1 353	>2560	>2560	90
c6 no doc 1ug IM	Post II	247	620	247	<20
c6 no doc 1ug IM	Post III	411	878	748	<20
2g2 no doc 10ug IM	Post II	>320	>2560	>2560	>2560
2g2 no doc 10ug IM	Post III	>2560	>2560	>2560	1407
2g2 no doc 1ug IM	Post II	>2560	>2560	>2560	119
2g2 no doc 1ug IM	Post III	>2560	>2560	>2560	348
2gecoN1b-1 no doc 10ug IM	Post II	>2560	>2560	>2560	1162
2gecoN1b-1 no doc 10ug IM	Post III	>2560	>2560	>2560	1213
2gecoN1b-1 no doc 1ug IM	Post II	1 151	>2560	1 696	22
2gecoN1b-1 no doc 1ug IM	Post III	2 220	>2560	1 947	135
c6 doc 10ug IM	Post II	308	248	341	<20
c6 doc 10ug IM	Post III	189	104	400	<20
c6 doc 1ug IM	Post II	33	43	63	<20
c6 doc 1ug IM	Post III	NC (>20)	24	156	<20
2g2 doc 10ug IM	Post II	NC (>20)	25	360	<20
2g2 doc 10ug IM	Post III	201	<20	647	<20
2g2 doc 1ug IM	Post II	275	<20	299/644	<20
2g2 doc 1ug IM	Post III	237	<20	728	<20
2gecoN1b-1 doc 10ug IM	Post II	573	31	685	<20
2gecoN1b-1 doc 10ug IM	Post III	NC (>40)	21	1 140	<20
2gecoN1b-1 doc 1ug IM	Post II	261	NC	118	<20
2gecoN1b-1 doc 1ug IM	Post III	348	NC	692	<20

Clearly, the presence of L3 (2g2) or intermediate (2gecon1b-1) epitope induces cross-bactericidal antibodies, while blebs from truncated LPS strain (C6) induce lower level of cross-reacting antibodies. This was particularly illustrated when 1µg of OMV was injected.

Moreover, as shown with OMV purified with DOC, reducing the LPS content of blebs reduces the induction of cross-bactericidal antibodies. Aside from increased LPS, it is possible that DOC free blebs may also advantageously retain some proteins losely interacting with the OMVs such as lipoproteins.

Example 3: Mutation of the *lgtG* gene to give constitutive expression of LgtG – the *lgtG"fixed"* mutant

Using strain *Neisseria meningitidis* strain 35E (L2 immunotype typing strain) as a template primer pair Lg1: 5'-ATG AAG CTC AAA ATA GAC ATT G-3' and Lg21: 5'- ATC TGC GGG CGG CGG CGC GAC TTG GAT-3', and primer pair LGdel18: 5'-GAA TTC GGA TCC AAC TGA TTG TGG CGC ATT CC-3' and

Lg2UP: 5'-TGC CGT CTG AAG ACT TCA GAC GGC TTA TAC GGA TGC CAG CAT GTC-3' (underlined sequence denotes a *Neisseria meningitidis* uptake sequence) were used to produce two PCR products. These products were purified and then used in splice overlap PCR with primers Lg 1 and Lg2UP to produce a final product that was cloned into the pGEM-T Easy vector (Promega). The resulting plasmid, pL2+, was sequenced to confirm that the wild type sequence of 11C in the wild type polyC tract of *lgtG* had been replaced with 5'-CGCCGCCGCCC-3'. The sequence of the *lgtG* coding sequence in the region of the mutation is shown in figure 3.

Transformation of strain MC58ϕ3*lgtAG2* with pL2+ to transfer the *lgtG"fixed"* mutation to the chromosome.

In order to transform the *lgtG"fixed"* mutation and detect the LPS phenotype with immunocolony-blot screening it was necessary to create a strain that was fixed "off" expression for LgtG. A kanamycin cassette from pUK4kan was clones into the *Xcm*I site of pL2+. The resulting plasmid, plgtG::kan, was used to transform 2G2 (see above) to kanamycion resistance and the correction position of the *lgtG::kan* allele was confirmed by PCR using primers Lg1 and Lg4 5'-AACCGTTTTCCTATTCCCAT-3', followed by nucleotide sequencing with the same primers. The resulting strain, ϕ3lgtA2GlgtG::kan-3, had the genotype: MC58 parent strain; *siaD::ery lgtAG2 lgtG::kan.* This strain was then transformed with plasmid pL2+ and screened for colonies with an L2 phenotype and screen by colony-immuno blots (Mn 42F12.32). Positive colonies were picked and tested for by both kanamycin sensitivity and PCR using primers Lg1 and Lg8 5'-CAC CGA TAT GCC CGA ACT CTA-3' followed by sequencing with primer Lg5 5'-CAC CGC CAA ACT GAT TGT-3' to confirm the *lgtG"fixed"* mutation had replaced the *lgtG::kan* allele. The resulting strain ϕ3lgtA2GlgtGL2+ has the genotype: MC58 parent strain; *siaD::ery lgtAG2 lgtG"fixed".*

We Claim:

1. A process of making a genetically engineered meningococcal strain comprising the step of genetically engineering a meningococcal strain with phase-variable LOS expression, to render LOS expression non-phase variable.

2. The process of claim 1, wherein the non-phase variable LOS has exclusively an L2 immunotype.

3. The process of claim 2, wherein the genetic engineering step comprises the elements of fixing the expression of both the lgtA and lgtG gene products such that expression of full-length, functional product may not be switched off by phase variation.

4. The process of claim 3, wherein the expression of either or both of lgtA and lgtG gene products is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the respective gene whilst maintaining the open-reading frame in frame.

5. The process of claim 4, wherein the homopolymeric G tract in the lgtA open-reading frame is reduced to 8, 5 or, preferably, 2 consecutive G nucleotides.

6. The process of claim 4 or 5, wherein the homopolymeric C tract in the lgtG open-reading frame is reduced to 8, 5 or, preferably, 2 consecutive C nucleotides.

7. The process of claims 3-6, wherein the expression of lgtA gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

8. The process of claims 3-7, wherein the expression of lgtG gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: one or more CCC codons encoding Proline is changed to any other codon encoding Proline, or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

9. The process of claim 7 or 8, wherein 2, 3 or 4 codons in the homopolymeric tract are changed, preferably to encode the identical amino acid.

10. The process of claim 3, wherein the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the respective gene to 2 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of lgtG gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtG gene such that: 1, 2 or 3 CCC codons encoding Proline is changed to any other codon encoding Proline, or a codon encoding a conservative mutation, and/or the GCC codon encoding Alanine is changed to any other codon encoding Alanine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

11. The process of claim 1, wherein the non-phase variable LOS has exclusively an L3 immunotype.

12. The process of claim 11, wherein the genetic engineering step comprises the elements of fixing the expression of the lgtA gene product such that expression of full-length, functional product may not be switched off by phase variation, and permanently downregulating the expression of functional gene product from the lgtG gene.

13. The process of claim 12, wherein the expression of lgtA gene product is fixed by reducing the length of the homopolymeric nucleotide tract within the open-reading frame of the gene whilst maintaining the open-reading frame of the gene in frame.

14. The process of claim 13, wherein the homopolymeric G tract in the lgtA open-reading frame is reduced to 8, 5 or, preferably, 2 consecutive G nucleotides.

15. The process of claims 12-14, wherein the expression of lgtA gene product is fixed by changing the sequence of the homopolymeric nucleotide tract within the open-reading frame of the lgtA gene such that: one or more GGG codons encoding Glycine is changed to any other codon encoding glycine, or a codon encoding a conservative mutation, and/or the TCG codon encoding Serine is changed to any other codon encoding Serine, or a codon encoding a conservative mutation, whilst maintaining the open-reading frame of the gene in frame.

16. The process of claims 12-15, wherein the expression of functional gene product from the lgtG gene is switched off.

17. The process of claim 12, wherein the expression of the lgtA gene product is fixed by reducing the length of the homopolymeric G nucleotide tract within the open-reading frame of the respective gene to 2 consecutive G nucleotides whilst maintaining the open-reading frame of the gene in frame, and the expression of functional gene product from the lgtG gene is switched off by deleting all or part of the promoter or open-reading frame of the gene.

18. The process of claims 2-17, wherein the genetic engineering step comprises the element of permanently downregulating the expression of functional gene product from the lgtC gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter or open-reading frame of the gene.

19. The process of claims 2-18, wherein the genetic engineering step comprises the element of permanently downregulating the expression of functional gene product

from the lgtB or lgtE gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter or open-reading frame of the gene.

20. The process of claim 19, wherein the genetic engineering step comprises the element of permanently downregulating the expression of functional gene product from the siaD gene, preferably by switching the gene off, most preferably by deleting all or part of the promoter or open-reading frame of the gene.

21. The process of claims 2-20, wherein the genetic engineering step comprises the element of permanently downregulating the expression of functional gene product from the msbB and/or htrB genes, preferably by switching the gene off, most preferably by deleting all or part of the promoter or open-reading frame of the gene.

22. A process of isolating L2 LOS comprising the steps of producing a genetically engineered meningococcal strain with a fixed L2 immunotype by the process of claims 2-10, 18-21; and isolating L2 LOS from the resulting strain.

23. The process of claim 22, comprising the additional step of conjugating the L2 LOS to a carrier comprising a source of T-cell epitopes and/or the step of presenting the L2 LOS in a liposome formulation.

24. A process of isolating meningococcal blebs having an L2 LOS immunotype, comprising the steps of producing a genetically engineered meningococcal strain with a fixed L2 immunotype by the process of claims 2-10, 18-21; and isolating blebs from the resulting strain.

25. The process of claim 24, where the step of isolating blebs is carried out either by extraction with a buffer containing no more than 0.1% deoxycholate, or by isolation without the use of a deoxycholate extraction step.

26. The process of claim 24 or 25, comprising the additional step of conjugating the L2 LOS to an outer membrane protein also present in the blebs.

27. A process of isolating L3 LOS comprising the steps of producing a genetically engineered meningococcal strain with a fixed L3 immunotype by the process of claims 11-17, 18-21; and isolating L3 LOS from the resulting strain.

28. The process of claim 27, comprising the additional step of conjugating the L3 LOS to a carrier comprising a source of T-cell epitopes and/or the step of presenting the L3 LOS in a liposome formulation.

29. A process of isolating meningococcal blebs having an L3 LOS immunotype, comprising the steps of producing a genetically engineered meningococcal strain with a fixed L3 immunotype by the process of claims 11-17, 18-21; and isolating blebs from the resulting strain.

30. The process of claim 29, where the step of isolating blebs is carried out either by extraction with a buffer containing no more than 0.1% deoxycholate, or by isolation without the use of a deoxycholate extraction step.

31. The process of claim 29 or 30, comprising the additional step of conjugating the L3 LOS to an outer membrane protein also present in the blebs.

32. A process of making an immunogenic composition comprising the steps of producing isolated L2 LOS by the process of claims 22-23 or producing isolated meningococcal blebs having an L2 LOS immunotype by the process of claims 24-26, and formulating said L2 LOS or blebs with a pharmaceutically acceptable excipient.

31. A process of making an immunogenic composition comprising the steps of producing isolated L3 LOS by the process of claims 27-28 or producing isolated meningococcal blebs having an L3 LOS immunotype by the process of claims 29-31, and formulating said L3 LOS or blebs with a pharmaceutically acceptable excipient.

32. A process of making a multivalent immunogenic composition comprising the steps of producing isolated L2 LOS by the process of claims 22-23 or producing isolated meningococcal blebs having an L2 LOS immunotype by the process of claims

24-26, producing isolated L3 LOS by the process of claims 27-28 or producing isolated meningococcal blebs having an L3 LOS immunotype by the process of claims 29-31, and mixing said L2 and L3 components together along with a pharmaceutically acceptable excipient.

ABSTRACT

The present invention relates to the field of neisserial vaccine compositions, their manufacture, and the use of such compositions in medicine. More particularly it relates to processes of making novel engineered meningococcal strains which are less phase variable in terms of their LOS immunotype, and from which novel LOS subunit or meningococcal outer-membrane vesicle (or bleb) vaccines can be derived.

Figure 1: L3 and L2 immunotypes (H44/76 , MC58 strains)

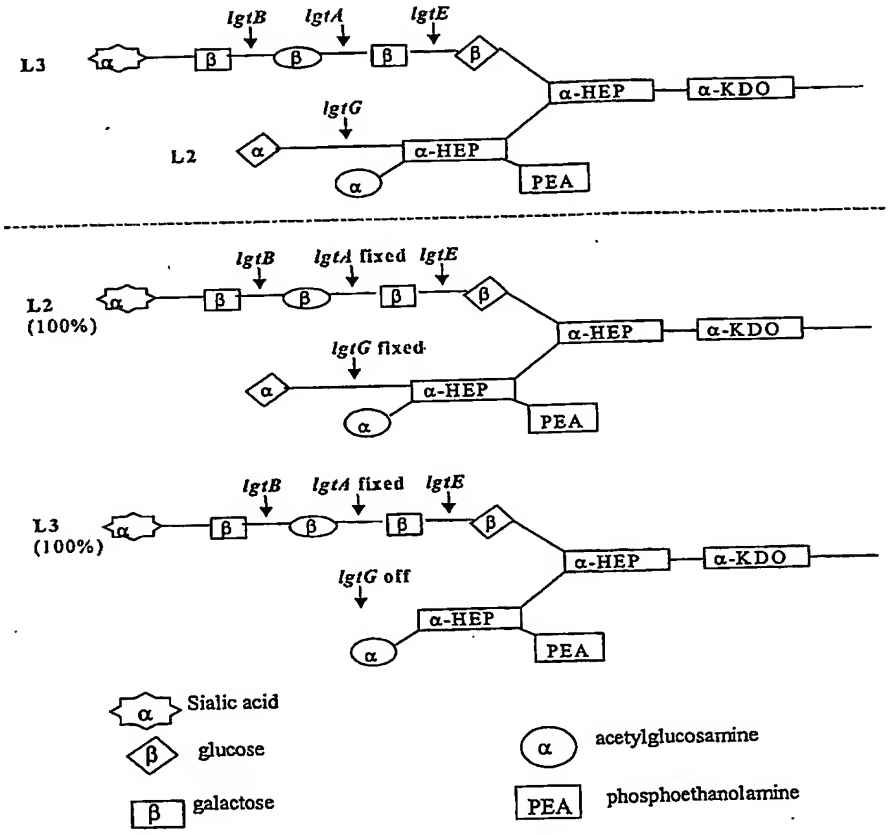
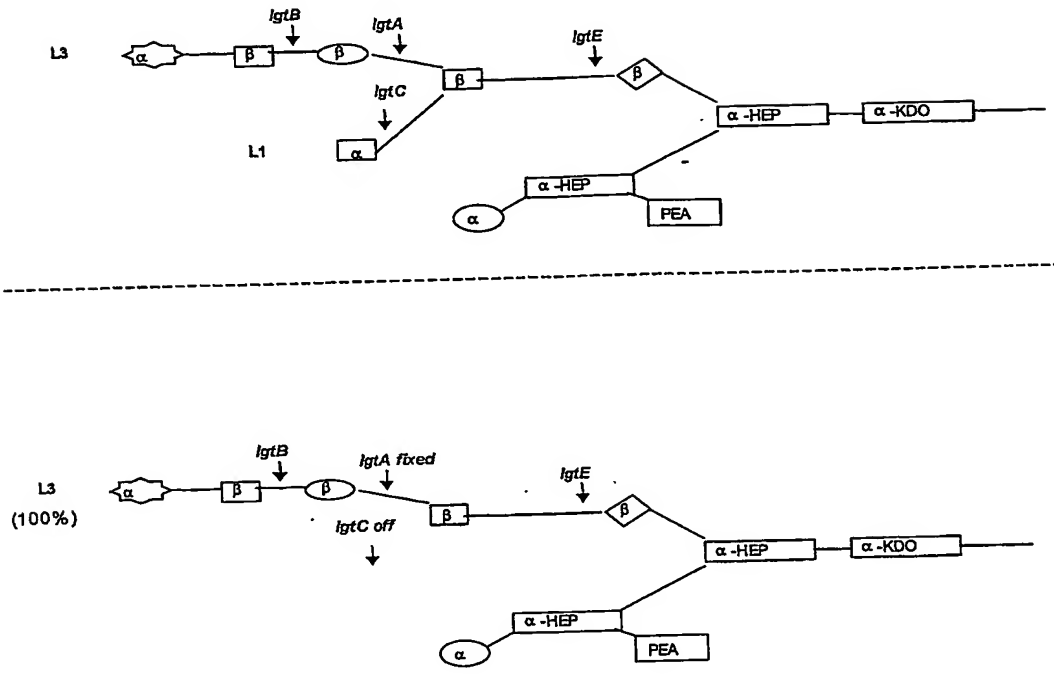


Figure 2: L3 and L1 immunotypes (e.g. 126E strain)



35E ATGAAGCTCAAAATAGACATTGCAACCAGCAACTTCAAACACGGCGGCGGCACGGAACGCTACACATTGGA
lgtGF ATGAAGCTCAAAATAGACATTGCAACCAACAACTTCAAACACGGCGGCGGCACGGAACGCTACACATTGGA

35E TTTGGTAAAGGGTCTGAACAGACAAAACATCACACCGGCCGTTTATGCGACGAAATTTGATCACGGCATTC
lgtGF TTTGGTAAAGGGTCTGAACAGACAAAACATCACACCGGCCGTTTATGCGACGAAATTTGATCACGGCATTC

35E CTGAATACGCCATGATCGAACCCCATCTTGTCGATCAACACCGGACGCTGAAAAAACTACGCCCATTCCTC
lgtGF CTGAATACGCCATGATCGAACCCCATCTTGTCGATCAACACCGGACGCTGAAAAAACTACGCCCATTCCTC

35E TTTTCAAGCCGGCTCACTCAAACCAGAAAAAACAGTGCCGCCAAACTGATTGCCTGCCACCACGCCGATTA
lgtGF TTTTCAAGCCGGCTCACTCAAACCAGAAAAAACAGTGCCGCCAAACTGATTGCCTGCCACCACGCCGATTA

35E CGCCGACCTCCTCATCTGCGGCGGCACACACTTGGGCTACCTGCACCATATGGCGCAAAAACCGAACCTGC
lgtGF CGCCGACCTCCTCATCTGCGGCGGCACACACTTGGGCTACCTGCACCATATGGCGCAAAAACCGAACCTGC

35E TCGACCGCCTCGCCATACGCCGTAACCGCAGCAACTACGCCACCGCCAAACTGATTATGGCGCATTCCCAT
lgtGF TCGACCGCCTCGCCATACGCCGTAACCGCAGCAACTACGCCACCGCCAAACTGATTATGGCGCATTCCCAT

35E ATGATGCGGTGCGAACTGGTCGGACTGTACGGCGTTCCCCCTGAAAGAATCCAAGTCGCCCCCCCCCCCGC
lgtGF ATGATGCGGTGCGAACTGGTCGGACTGTACGGCGTTCCCCCTGAAAGAATCCAAGTCG**CGCCGCCGCCCC**GC

35E AGATACGGAACGCTTCTTCCCGCAACCCGGAGAAGCTGCCGACCTGCGCGCCAAATACGGCTTTGCCGACT
lgtGF AGATACGGAACGCTTCTTCCCGCAACCCGGAGAAGCTGCCGACCTGCGCGCCAAATACGGCTTTGCCGACT

35E ATGAAACCGTTTTCCTGTTCCCATCGACCGGCCACACGCGCAAAGGTCTGGAACTGCTTGCCGACTTTTTC
lgtGF ATGAAACCGTTTTCCTGTTCCCATCGACCGGCCACACGCGCAAAGGTCTGGAACTGCTTGCCGACTTTTTC

35E GAACATACCGGCCTGCCCGTCAAGCTCGCCGTTGTCGGCTC
lgtGF GAACATACCGGCCTGCCCGTCAAGCTCGCCGTTGTCGGCTC

Figure 3. Alignment of nucleotide sequence of the wild-type sequence of the *lgtG* gene of *Neisseria meningitidis* strain 35E and the *lgtG* "fixed" mutation (underlined, bold) contained on plasmid pL2+. Also shown is an *Xcm*I restriction endonuclease cleavage site used to construct an *lgtG::kan* mutant.